Protein Insertion into the Membrane of the Endoplasmic Reticulum: The Architecture of the Translocation Site

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In mammalian cells, most membrane proteins are inserted cotranslationally into the membrane of the endoplasmic reticulum (ER), and secretory proteins are translocated across this membrane (for review, see Rapoport 1992). These processes are initiated in the cytoplasm by binding of the signal sequence of the nascent polypeptide chain to the signal recognition particle (SRP) (Walter and Blobel 1981). The resulting ribosome nascent chain SRP complex is then targeted to the ER membrane by an interaction with the membrane-bound SRP receptor (docking protein) (Gilmore et al. 1982; Meyer et al. 1982). At the membrane, the signal sequence is released from SRP in a GTP-requiring step, and the nascent polypeptide is transferred into the ER translocation site (Connolly and Gilmore 1989).

The translocation site is thought to comprise an aqueous channel through which the nascent polypeptide traverses the membrane (Blobel and Dobberstein 1975). Evidence for such a channel comes from several lines of research: Electrophysiological studies revealed the presence of large aqueous pores in rough ER membranes (Simon and Blobel 1991); membrane-inserted nascent polypeptides could be removed from the membrane by agents perturbing protein-protein interactions (Gilmore and Blobel 1985); and fluorescent probes incorporated into a translocating polypeptide reported a hydrophilic surrounding of nascent chains traversing the membrane (Crowley et al. 1993, 1994). Membrane proteins forming the protein-conducting channel were identified by cross-linking experiments and reconstitution studies (Wiedmann et al. 1987; High et al. 1991, 1993a,b; Görlich et al. 1992a). A central component is the multispanning α subunit of the Sec61p complex (Görlich et al. 1992b). This protein could be cross-linked to membrane-inserted secretory as well as type I and type II membrane proteins and therefore is thought to form the core of the protein-conducting channel (High et al. 1993b; Mothes et al. 1994). Apart from the Sec61p complex, only two other components, the TRAM protein (Görlich et al. 1992a) and the SRP receptor, are essential for translocation of polypeptides into reconstituted proteoliposomes (Görlich and Rapoport 1993).

The mechanism by which nascent secretory and membrane proteins insert into the membrane is not known. Nascent secretory proteins and nascent type II membrane proteins are thought to adopt a loop-like configuration, with the amino terminus remaining in the cytoplasm and the growing carboxy-terminal part being continuously translocated across the membrane (Shaw et al. 1988; High and Dobberstein 1992). In the case of a secretory protein, cleavage of the signal sequence then releases the protein into the ER lumen. In type II membrane proteins, a signal anchor sequence functions in targeting and membrane insertion. The signal anchor sequence is not cleaved by the signal peptidase and anchors the protein in the lipid bilayer such that the protein spans the membrane with its amino terminus in the cytoplasm and the carboxyl terminus in the ER lumen.

IDENTIFICATION OF LIPIDS IN THE TRANSLOCATION SITE BY SITE-SPECIFIC CROSS-LINKING

Because signal and signal anchor sequences are hydrophobic in nature, it has been postulated that they interact with lipids at some stage of membrane insertion and anchoring (Wickner 1979; Engelmann and Steitz 1981). This is particularly expected in the case of a type II membrane protein that becomes anchored in the lipid bilayer. To identify lipids surrounding a membrane-spanning segment, we have used the type II membrane protein invariant chain in a cross-linking study. The essential elements of the assay system are as follows: the insertion of a cross-linker into the nascent polypeptide chain (Brunner 1993); the formation of a stable translocation intermediate in which the signal anchor sequence spans the membrane (Gilmore et al. 1991); the light-induced cross-linking and the analysis of the cross-link product by high-resolution SDS-PAGE (Martoglio et al. 1995).

For site-specific photocrosslinking (High et al. 1993b), the photoactivatable, carbene-generating amino acid L-4′-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]-phenylalanine ([Tmd]Phe) (Baldini et al. 1988) is biosynthetically incorporated at single, selected positions of the nascent polypeptide chain. The incorporation of (Tmd)Phe is mediated by a chemically aminoacylated suppressor tRNA and directed by an amber (stop) codon. Translation only proceeds if the suppressor tRNA has successfully incorporated the photoactivatable amino acid (Tmd)Phe into the nascent polypeptide (High et al. 1993b). Consequently, all completed translation products contain the cross-

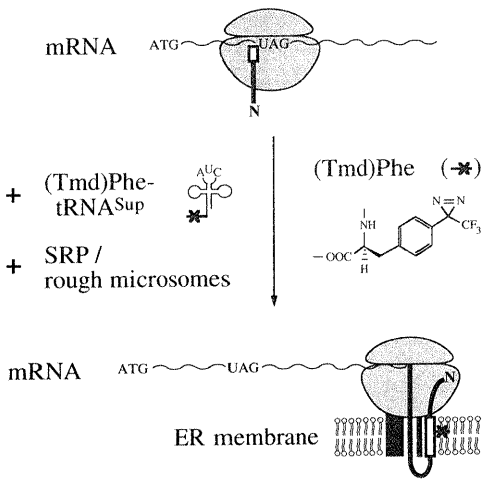

Figure 1. Site-specific photocrosslinking of translocation intermediates. In an in vitro translation system, truncated mRNAs are translated in the presence of an amber suppressor tRNA chemically charged with the cross-linking reagent (Tmd)Phe (asterisk). In the presence of rough microsomal vesicles, ribosomes attach to the membrane and nascent chains insert into the translocation site but are not released from the ribosome. Upon UV-irradiation, the cross-linking reagent covalently links to neighboring molecules.

linking reagent at only one position (Fig. 1). This system of site-specific photocrosslinking therefore allows quantification of cross-linking efficiencies. Such a quantification is important for determining the degree to which the nascent polypeptide interacts with the various constituents of membranes, protein, lipid, or solvent. To detect proteins and also lipids, it is important that the cross-linking reagent is able to link to the neighboring molecules irrespective of their chemical nature, including aliphatic side chains. The diazirin we use as a cross-linking reagent yields (after light activation) a carbene that fulfills these requirements (Brunner 1989).

When proteins are cross-linked to translocating polypeptides, the resulting cross-link products are easily detected by SDS-PAGE due to a decrease in the electrophoretic mobility. The reduction in mobility reflects the size of the component that is cross-linked to the nascent chain. This has been demonstrated for the interaction of the signal sequence with SRP54 and with the Sec61α protein (Krieg et al. 1986; Kurzchalia et al. 1986; High et al. 1993a,b; Mothes et al. 1994). Because lipids are small molecules, their identification is rather difficult. A detectable increase in the apparent molecular weight cannot always be expected. Nascent chains have to be small (M_r <30 kD), and a gel system must be used in which differences in molecular size of 0.5 kD can be detected. That the cross-link product contains a lipid molecule can be shown by treatment with phospholipase A_2 or chemically, by alkaline hydrolysis (Martoglio et al. 1995). Both treatments result in cleavage of the ester bond between the fatty acyl side chain and the polar headgroup.

LIPIDS IN THE TRANSLOCATION SITE

To characterize the molecular environment of a translocating polypeptide, translocation intermediates of defined length have to be prepared (Fig. 1). Transmembrane-arrested translocation intermediates were obtained by truncating mRNAs in their coding sequence and translating them in the presence of microsomal membranes. Under these conditions, the nascent chain is not released from the ribosome at the 3′ end of the mRNA because a termination codon is lacking (Gilmore et al. 1991).

Type II Membrane Proteins

To study the molecular environment of a signal anchor sequence during different stages of insertion into the membrane of the ER, we incorporated the cross-linking reagent (Tmd)Phe via a suppressor tRNA into

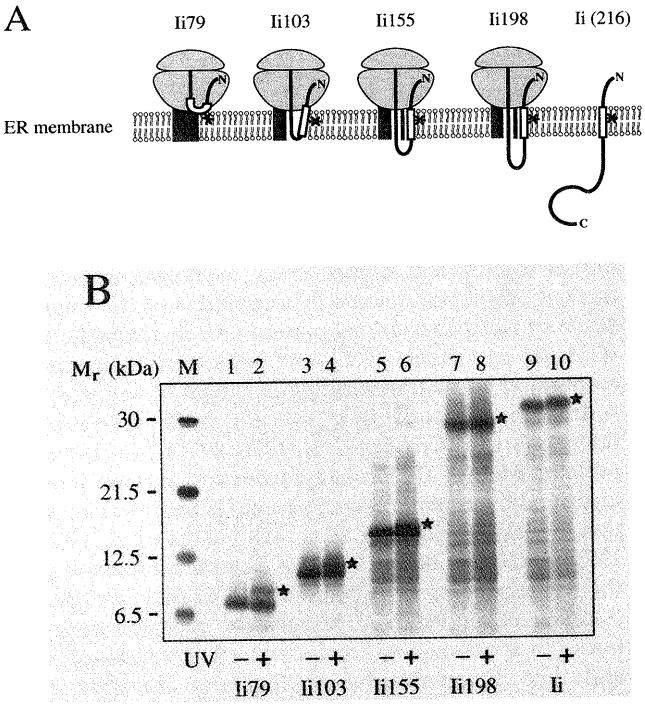

Figure 2. Photocrosslinking between a signal anchor sequence and interacting components. (*A*) Schematic illustration of translocation intermediates with chain lengths of 79, 103, 155, 198, and 216 amino acid residues. The signal anchor sequence is indicated by a square; the hydrophilic part as thick line. The site where (Tmd)Phe is incorporated as indicated by an asterisk. (*B*) Radiolabeled translocation intermediates are formed. One aliquot is directly analyzed (UV−) and in the other, cross-linking is induced by UV light (UV+). Proteins are then analyzed by SDS-PAGE and autoradiography. Cross-links to lipids are indicated by a star.

the center of the hydrophobic core of the signal anchor sequence. As a model protein we used the invariant chain (Ii), a 33-kD type II membrane protein. Nascent chains of increasing length (79, 103, 155, 198, and 216 amino acid residues) were inserted into the ER membrane (Fig. 2A). Upon cross-linking, the molecules contacting the signal anchor sequences were identified by analyzing the cross-link products. In Figure 2B, such an analysis by SDS-PAGE is shown. Even with the shortest insertion intermediate (79 amino acids), which is probably too short to completely span the membrane, cross-links to lipids could be detected. This may indicate that a type II membrane protein enters the membrane at a protein-lipid boundary with the signal anchor sequence contacting lipid molecules. With longer insertion intermediates (103 and more amino acid residues), up to about 50% cross-linking to lipids was observed (Martoglio et al. 1995). This probably reflects the fact that the signal anchor sequence is almost completely embedded in the lipid bilayer. In the hydrophobic phase of the membrane, about 50% quenching by solvent molecules can be expected (Brunner 1989).

Besides being cross-linked to lipids, the Ii signal anchor sequence was also cross-linked to the TRAM protein and to Sec61α with low efficiency. These cross-links to components of the protein-conducting channel were not found when Ii translocation intermediates were released from the ribosome by puromycin, indicating that they had left the proteinaceous environment of the ER translocation site (Martoglio et al. 1995). Contact with lipids was confined to the hydrophobic core region of the signal anchor sequence. When (Tmd)Phe was incorporated into the segment carboxy-terminally following the hydrophobic region, Ii translocation intermediates were only found to cross-link with low efficiency to Sec61α.

Secretory Proteins

Components contacting translocation intermediates of the secretory protein preprolactin (PPL) have been identified previously by using either trifluoroethyl diazirino benzoic acid (TDBA)-modified lysyl-$tRNA^{Lys}$ (Mothes et al. 1994) or (Tmd)Phe-$tRNA^{Sup}$ (High et al. 1993b). The signal sequence of PPL was found to be in contact with the TRAM protein and with Sec61α. Using the site-specific photocrosslinking approach described above, we also found contact with lipid molecules (Martoglio et al. 1995). Cross-linking to lipids ranged from 5% to 17%, significantly lower than found with the signal anchor sequence of Ii. This might indicate that the PPL signal sequence has only limited access to lipid molecules, which is consistent with the shorter hydrophobic core region (12 hydrophobic residues) compared to the Ii signal anchor sequence (26 hydrophobic residues) (Fig. 3).

TWO DISTINCT SITES IN THE TRANSLOCATION COMPLEX: THE AQUEOUS TRANSLOCATION CHANNEL AND THE HYDROPHOBIC SIGNAL SEQUENCE RETENTION SITE

Nascent secretory proteins and nascent type II membrane proteins are thought to enter the ER translocation site in a loop-like configuration (Shaw et al. 1988). In this loop, two segments of the nascent polypeptide span the membrane, the signal or signal anchor sequence that is retained in the membrane, and the translocating portion of the growing polypeptide chain (Fig. 3A). These functionally different segments are embedded in a different molecular environment. Signal and signal anchor sequences are both in contact with lipids at any tested stage of their membrane insertion (Martoglio et al. 1995). The translocating portion of nascent polypeptide chains is in a hydrophilic environment and in contact with the Sec61p complex. This is concluded from several lines of evidence. Mothes and coworkers (1994) have systematically probed the hydrophilic portion of translocating nascent PPL chains by a site-specific photocrosslinking approach. These authors found that Sec61α is the major ER membrane protein in proximity to the translocating polypeptide. Cross-links to lipids were not reported. The results of Mothes and coworkers are consistent with our observation that the hydrophilic part of membrane-inserted Ii is in contact with Sec61α but not with lipids (Martoglio et al. 1995). The efficiency of cross-linking to Sec61α was rather low (~3%) if compared to cross-linking efficiencies obtained when the interaction between two reaction partners is tight (e.g., nascent chain x SRP54, ~50%). This low cross-linking efficiency most likely reflects random collision of the nascent chain with the "wall" of the protein-con-

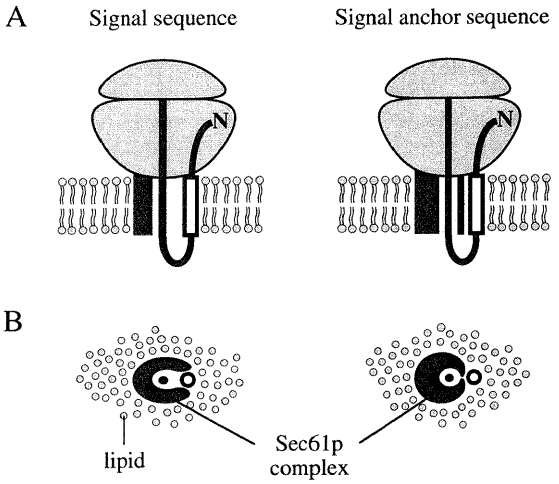

Figure 3. Schematic drawing of the proposed localization of membrane-inserted nascent chains in the translocation site. (*A*) Side view; (*B*) top view. The signal/signal anchor sequence is shown by a square in *A* and an open circle in *B*.

ducting channel formed by the Sec61p complex and quenching of the UV-activated cross-linker by water molecules (Brunner 1989). An aqueous environment of the translocating portion of the nascent polypeptide chain has been reported previously by using fluorescent dyes incorporated into the hydrophilic portion of nascent polypeptide chains (Crowley et al. 1993, 1994).

Our finding that proteins and lipids line distinct regions of the membrane-inserted polypeptide could also indicate that nascent chains enter the protein-conducting channel at the protein-lipid interface. The degree to which the signal and signal anchor sequence contact lipid may depend on the hydrophobicity of these sequences (Fig. 3B). Signal anchor sequences with extended hydrophobic core regions (>20 residues) are exposed more toward the lipid bilayer than signal sequences with short hydrophobic cores (7–15 residues) (von Heijne 1985, 1986). Differences in the environment of signal and signal anchor sequences during their membrane insertion have also been proposed by Nilsson et al. (1994). These authors found that the position of a signal sequence or a signal anchor sequence relative to the oligosaccharyltransferase is different. Furthermore, Nilsson and coworkers showed that the signal peptidase has access to signal sequences with hydrophobic core regions of less than 15 amino acid residues but not to those with extended (>17 residues) hydrophobic cores. Thus, it appears that the arrangement of the components in the translocation complex is determined by the hydrophobic properties of signal and signal anchor sequences of nascent polypeptides.

SUMMARY AND OUTLOOK

Different methodological approaches have given a first insight into the architecture of the protein-conducting channel in the ER membrane: Electrophysiology revealed aqueous channels (Simon and Blobel 1991); fluorescent probes showed an aqueous surrounding of the translocating polypeptide (Crowley et al. 1993, 1994); site-specific cross-linking revealed lipids in contact with signal and signal anchor sequences (Martoglio et al. 1995); various cross-linking approaches demonstrated that proteins of the Sec61p complex line the protein-conducting channel (High et al. 1991, 1993a,b; Görlich et al. 1992b; Mothes et al. 1994); and reconstitution studies revealed that the Sec61p complex is an essential element of the protein translocation site (Görlich and Rapoport 1993).

Besides the Sec61p complex, there are several other proteins associated with the translocation complex, including TRAMp (Görlich et al. 1992a), TRAP (Hartmann et al. 1993), and the RAMPs (Görlich et al. 1992b). They have been identified by their tight association with the ribosome nascent chain complex. These proteins may adapt the translocation site to the various substrates (e.g., different types of membrane proteins) or provide the structural basis for regulation of protein translocation. Proteins involved in the modification of translocating polypeptides like signal peptidase, signal peptide peptidase, and chaperones may only interact transiently with the translocation complex.

The architecture of the protein-conducting channel may change according to (1) the stage of insertion or translocation and (2) the type of protein inserted or translocated. Little is known about the initial stage of protein insertion into the ER membrane. We envision insertion into a protein-lipid interface (Martoglio et al. 1995). After insertion, a signal sequence is cleaved from the membrane-inserted nascent chain and is further proteolytically trimmed by a signal peptide peptidase. Fragments of the signal peptide are then released to the cytosol and into the ER lumen (Henderson et al. 1992; Lyko et al. 1995). How the cleaved signal peptide exits the translocation site is not known. Whether the protein-conducting channel closes around an extended or partially folded translocating polypeptide is also unclear.

Differences in the molecular environment of inserting secretory proteins and inserting type II membrane proteins have been reported (Nilsson et al. 1994; Martoglio et al. 1995). The lengths of the hydrophobic core region of their signal and signal anchor sequences are usually different (von Heijne 1985, 1986). Thus, these sequences insert into a protein or lipid environment to a different degree. This has been shown for two examples, but further investigations are needed before generalizations can be made.

In type I membrane proteins with a cleavable signal sequence, a stop transfer sequence retains these proteins in the membrane. Assuming that a stop transfer sequence enters the protein-conducting channel, its exit into the lipid bilayer needs a lateral opening of the channel. A similar requirement exists for proteins spanning the membrane several times. Membrane-spanning segments must gain access to the lipid bilayer. Exit to the lipid bilayer may occur sequentially. However, it is also conceivable that the protein-conducting channel, like a chaperone, accommodates partially folded peptide segments and releases folded domains comprising several transmembrane stretches into the lipid bilayer. In this view, translocation of proteins across the ER membrane closely relates to protein folding with two options, to go to the lipid bilayer or to go to the ER lumen.

ACKNOWLEDGMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB-352) and the Human Frontier Science Program Organization.

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